ISSN: 0974-5343
IJMST (2011), 4(3):13-22

Characterization of isoniazid-resistant mutant (S315R) of catalase-peroxidase, KatG, from *Mycobacterium tuberculosis*

Dr. A. Nusrath Unissa[1], Dr. N. Selvakumar[2], Dr. Sujatha Narayanan[3]

1,2 Department of Bacteriology, National Institute for Research in Tuberculosis, Indian Council of Medical Research, Chennai, INDIA
3Department of Immunology, National Institute for Research in Tuberculosis, Indian Council of Medical Research, Chennai, INDIA
nusrathunissa@gmail.com, selvakumar.nagamiah@gmail.com

ABSTRACT:
Catalase-peroxidase, KatG, plays a key role in activating the prodrug isoniazid - an important drug in the anti-tuberculosis therapy. Mutation in *katG* gene is a major mechanism of INH resistance in *Mycobacterium tuberculosis*. In this study, the characterization of KatG mutant S315R and wild type (WT) was performed using rDNA technology. The catalytic turnover (k_{cat}/K_m) in the mutant was decreased by 20% for catalase activity whereas the peroxidase activity was found to decrease by 62% and 61% with ABTS and INH compared to WT KatG respectively. The kinetic data indicates that the mutant activity towards INH was reduced considerably compared to WT. The results suggest that lesser activity in the mutant (S315R) could be due to the steric hindrance mediated by the bulkier guanido group present in the Arg residue instead of simpler hydroxyl group in the Ser residue. This could have induced conformational change in the mutant protein and leads to altered binding of Heme, eventually affects the binding of INH causing resistance.

Key Words: *Mycobacterium tuberculosis*, INH resistance, KatG, Mutant, Cloning, expression, characterization

INTRODUCTION

Tuberculosis (TB) still remains as a major health problem and a leading cause for mortality. The increase in MDR-TB (strains resistant to at least two of the first-line TB drugs - isoniazid [INH] and rifampicin [RIF]) and the recent emergence of extensively drug resistant (XDR-TB) which is defined as MDR-TB that is resistant to any fluoroquinolone, and at least one of three injectable second-line drugs [capreomycin, kanamycin, and amikacin] has further worsened the situation. Drug resistance in TB is essentially a potential threat to the TB control programmes. However, isolates of *M. tuberculosis* resistant to INH are seen with increasing frequency (1 in 10^6) compared to other drugs [1].

INH is the cornerstone of treatment for drug-susceptible and latent TB infection, acting as a principle component in the current six-month short course chemotherapy. INH is a pro-drug that requires cellular activation by KatG protein to its active form before exerting its toxic effect on the bacillus. The first mechanistic insight of INH was revealed in 1954 when Middlebrook and others noted an inverse relationship between catalase and peroxidase (CP) activity and INH resistance [2]. It has long been recognized that resistance of *M. tuberculosis* to INH correlates with the loss of CP activities in resistant strains [3]. The work of Zhang *et al*. (1992) was first to demonstrate the genetic basis for INH resistance connected with loss of CP in *M. tuberculosis* upon isolation, cloning, and sequencing of the *katG* gene [4]. The *katG* gene encodes an 80-kDa hemoprotein of 744 amino acids that has CP activity. KatGs are bifunctional heme enzymes that exhibit both catalase activity and broad-spectrum peroxidatic activity comparable with monofunctional peroxidases [5, 6].

There has been considerable interest to know the molecular basis of INH resistance which is not very well characterized, and mutations in several genes have been associated with it. Zhang et al. (1992) demonstrated that mutation in *katG* gene coding for KatG protein is a major mechanism of INH resistance in *M. tuberculosis*. Classical resistance to INH arises from deletions or point mutations in *katG*, which encodes KatG/CP. Using the crystal structure of CP, the effects of a number of point mutations located in the proposed INH binding site in the distal pocket can now be rationalized to some extent. Rouse *et al*. (1996) generated 13 mutants of *katG* using site-directed mutagenesis to alter the WT *katG* gene from *M. tuberculosis* at 13 codons previously shown to be mutated in INH^r clinical isolates [7]. Importantly, nine of the 13 variant amino acids were shown to confer INH resistance, including Arg104Leu, His108Gln, Asn138Ser, Leu148Arg, His270Gln, Thr275Pro, Ser315Thr, Trp321Gly, and Asp381Gly. These results were consistent with modeling studies suggesting that residues 104 and 108 are located at or near the enzyme catalytic site and residues 270, 275, and 315 participate in heme binding [8]. In another report, six mutants of KatG (Ala110Val, Ala139Pro, Ser315Asn, Leu619Pro, Leu634Phe, and Asp735Ala) were kinetically characterized [9]. In two other reports, four mutants of KatG (Cys20Ser, Thr275Pro, Leu587Met and Leu587Pro) and (Asn138Ser, Thr275Pro, Ser315Thr, and Asp381Gly) were characterized [10, 11]. Pym *et al*. (2002), has assessed the activity of three mutants of KatG Ala139Val, Thr275Pro, and Ser315Thr in relation to virulence [12]. Also in another study, the activity of a mutant Trp321Phe has documented [13].

Several workers from all over the world observed varying responses of the KatG mutants showing either

a decrease in or abolition of CP activities. Of these, the mutant S315T was studied extensively [11, 14-18]. The Ser to Thr mutation (AGC to ACC) in codon 315 of the *katG* gene is considered to be the most prevalent mutation, serving as a reliable marker for the detection of INH resistance [8] and the appearance was most frequent among the MDR strains [19]. However, this mutation was also reported to be associated with intermediate or high levels of resistance to INH (1 to 10 µg/ml) [20]. It has been reported that Ser315 was mutated to Asn, Iso, Arg, and Gly although the most frequently occurring mutation is to Thr [8]. Although the characterization of mutants with Ser to Asn and Gly substitution was already reported [9, 21] the characterization of other mutant's *viz.*, Ser to Arg and Iso was not yet carried out and therefore still remains an area to be explored. Hence, in the present study functional characterization for one of the clinical mutants Ser to Arg at codon 315 along with the WT was undertaken.

MATERIALS AND METHODS

Reagents

Molecular reagents associated with PCR amplification, purification, Sequencing was obtained from Amersham Biosciences, UK. Enzymes from New England Biolabs, pBAD/Thio-TOPO cloning kit from Invitrogen Inc. Chemicals such as ABTS, NBT, H_2O_2 were purchased from Sigma Aldrich, USA. Few other Molecular reagents were purchased from Bangalore Genei India Ltd. All other chemicals used were of the analytical grade obtained from Himedia and Qualigens Fine Chemicals, India.

Genomic DNA extraction

The genomic DNA of *M. tuberculosis* H37Rv and a clinical mutant with CGC (confirmed by DNA sequencing) at position 315 was extracted as described elsewhere [22].

Amplification

PCR was carried out using the primers to amplify the *katG* gene of 2.2 kb from both the genomic DNA. Amplification was performed using the mixture containing 1.5 µl of forward and reverse primers (10 pmol) each, 2.5 µl of deoxyribonucleoside triphosphates (dNTP) mix (2.5mM), 2.5 µl of 10X PCR buffer, 200 ng of template genomic DNA and 0.5-1.0 µl of *Taq* DNA Polymerase. A mixture of DNA polymerase containing *Taq* and a proofreading polymerase - Vent was used. The *Taq* polymerase was used in excess ratio to ensure the presence of 3´ A-overhangs on the PCR product. The amplification was performed in a thermal controller (MJ Research, USA) with 30 cycles (1 min at 95°C, 2.30 min at 65°C and 1 min at 72°C, followed by a final extension step at 72°C for 10 min) in the isolated genomic DNA. The primers of *katG* gene forward sequence (5- 5' GTG CCC GAG CAA CAC CCA CCC-3') and reverse sequence (5' CTC GAC AGG TTC GAC GTG CGC - 3') were used to generate a 2.2 kb fragment containing the S315R codon. The primers used were designed with the help of software CLONE MANAGER (V-4.0). The designing was performed cautiously by non-introduction of termination/stop codon (TGA) and considering GC content. Also, the Tm (melting temperature) was checked for optimization of PCR. The amplicons were purified using Qiagen purification system according to the manufacturer's instructions.

Cloning

The purified amplicons were cloned into pBAD/Thio-TOPO expression vector (Fig. 1). This plasmid vector provides a highly efficient and direct insertion of amplicons for soluble, regulated expression which is driven by the *ara*BAD promoter and protein purification in *E. coli*. The AraC gene product encoded on the pBAD/Thio-TOPO plasmid positively regulates this promoter. Recombinant proteins are expressed as fusions to His-Patch thioredoxin for high-level expression and purification.

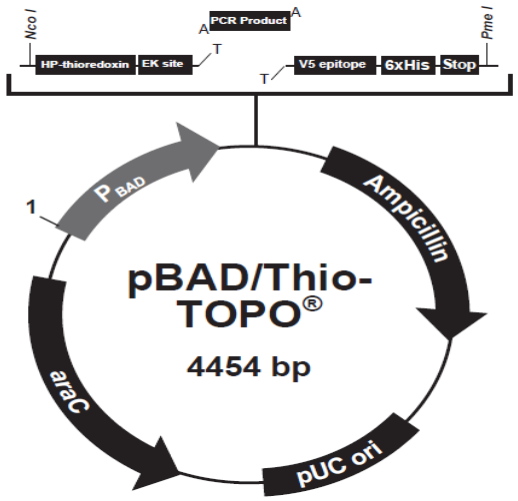

Fig.1: Map of pBAD/Thio-TOPO expression vector

The PCR expression vector pBAD/Thio-TOPO is a linear vector with single 3´ thymidine (T) overhangs for TA Cloning and Topoisomerase I bound to the vector (this is referred to as "activated" vector). *Taq* polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. This allows PCR inserts to ligate efficiently with the vector. The Topo cloning reaction mix consists fresh PCR product 4 µl, TOPO vector 1 µl and Salt solution 1 µl. The reaction mix was incubated for overnight at 16°C for ligation to occur.

Transformation

The host cell was provided with the vector system, *E. coli* strains, TOP 10 were used as cloning hosts. Genotype of TOP10 is F- *mcr*A δ(*mrr-hsd*RMS-

*mcr*BC) Φ80*lac*ZδM15 δ*lac*X74 *rec*A1 *ara*D139 δ(*araleu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G. For preparation of competent cells, 1 ml of overnight grown culture of TOP 10 cells was inoculated into 100 ml of fresh LB medium. The culture was incubated at 37°C with shaking till it reached an OD_{600} value of 0.3-0.4. The culture was kept at 4°C to arrest the growth. After 30 min, the cells were pelleted at 4°C and resuspended in 2 ml of TSS buffer. For long-term storage, the cells were frozen immediately in a dry ice-ethanol bath and stored at –70°C in 500 µl aliquots. For transformation of *E. coli*, 100 µl of competent cells was aliquoted into a pre-chilled polypropylene tube. The ligation mix was added followed by gentle mixing. The cell-DNA mixture was incubated on ice for 15 min. LB broth was added to a final volume of 1 ml and the cells were incubated at 37°C with shaking for an hr to allow expression of the ampicillin resistance gene. The transformants were selected using standard methods of plating the cells on selection plates containing ampicillin and/or X-gal.

Isolation of Plasmid DNA

The colonies from the grown cultures of the Petri plates were screened for recombinants by plasmid preparation. *E. coli* (TOP 10) with the plasmid was grown overnight in 10 ml of LB medium with 100 µg/ml ampicillin. The grown culture was subjected to isolation of plasmid by the alkaline lysis method [23]. The cells were pelleted, resuspended in TE buffer (pH 8.0) containing 10 mg/ml RNase, treated with alkaline SDS with 0.2N NaOH and 1% SDS and neutralized by adding ice-cold potassium acetate (3 M; pH- 4.8). After centrifugation of the sample at 10,000 rpm for 10 min at 4°C, the supernatant was transferred to a fresh tube and DNA precipitated by adding 0.7 vol/vol of isopropanol, washed with 70% ethanol; dried and dissolved in TE buffer.

Restriction Digestion

Recombinant plasmid DNA was analyzed for the presence of insert in proper orientation before proceeding with the expressions studies by restriction digestion (*NcoI*), an alternative simple and rapid method, in addition to the normal sequencing for checking the right conformation of the insert in the forward direction. Generally, 1-2 µl of recombinant DNA was incubated with 1X appropriate reaction buffer and 10-20 U of the respective restriction enzyme. The DNA-enzyme mixture was incubated for 2 hrs at 37°C [24].

Overexpression and conditional expression of katG

To confirm the expression of plasmid encoded *katG*, whole cell lysates of *E. coli* were prepared by growing the culture from individual colonies of the transformants in LB broth with ampicillin (100 µg/ml) at 37°C. The overnight grown cultures were sub cultured in fresh medium (1:100) to an OD_{600} of 0.6-0.8 and induced with 0.02% L-Arabinose at different time points of 1, 2, 3, 4, 5 hrs or overnight in LB broth. 1.5 ml of the culture was pelleted at 7000 rpm for 3 min and resuspended in 100 µl of lysis buffer. Lysate was centrifuged at 13,000 rpm to remove unlysed cells and debris. The supernatant was mixed with 6X SDS loading dye, denatured at 95°C. 40 µl of each sample was resolved in 10% SDS-PAGE gel.

Detection of recombinant KatG protein

SDS-PAGE was performed in slab gels in Tris buffer system, the running buffer was 1X Tris Glycine buffer. The samples were loaded under reducing conditions by boiling the sample in sample buffer for 5 min at 100°C. The proteins separated by the polyacrylamide gels were stained with CBB. After electrophoresis the gel was transferred to a plastic container and stained with the staining solution for an hr at room temperature with shaking. Then the stain was removed and destained by successive incubation in destainer till the background is clear.

Immuno-blotting

The immune-blotting was performed using anti-His-C-terminal-HRP antibody, the above gel was subjected to transfer on to nitrocellulose membrane to confirm the presence of the over expressed protein. The antibody was provided with Ni-NTA purification system, which enables to detect the recombinant protein, tagged with 6x-His residues at the C-terminal end of the target protein. The antibody coupled with enzyme HRP was raised against this C-terminal tagged 6x-His residues. Hence it specifically binds the target protein and in the process generates colour upon exposure to H_2O_2 for which the membrane was soaked in 100% of methanol for 10 min and equilibrated with transfer buffer.

Proteins were transferred on this membrane electrophoretically at 50 V for an hr in a blotting apparatus. Membrane was removed and incubated in 20 ml of blocking buffer consisting of PBST and 5% skimmed milk for an hr, then washed with 20 ml of PBST twice for 5 min. 10 ml (1:5000 dilution) of anti-His-C-terminal HRP antibody was added which allow one-step detection using H_2O_2 and DAB.

Purification of Recombinant KatG proteins

The purification of KatG proteins were carried out from the whole cell lysates of *E. coli* by growing cells in 5 ml, which then scaled up to 500 ml of LB broth containing 100 µg/ml ampicillin and 1ml of 20% L-Arabinose along with ferric chloride for the proper functioning of the heme. The grown cultures were sonicated using a sonicator equipped with a microtip with six 30-sec bursts at high intensity with a 30 sec cooling period between each burst in the presence of lysis buffer on ice. The lysate was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was used for purification process.

a) Probond resin Ni-NTA Agarose (resin) is used for purification of recombinant proteins expressed in bacteria, insect, and mammalian cells from any 6x-His-tagged vector. The resin exhibits high affinity and selectivity for 6x-His-tagged recombinant fusion proteins. It is precharged with Ni2+ ions and appears blue in colour. It is provided as 50% slurry in 30% ethanol. Proteins were purified under native and denaturing conditions using this resin. Proteins bound to the resin were eluted with competition with imidazole. The resulting proteins were ready for use in target applications.

b) Preparing Ni-NTA Column: The Ni-NTA Agarose (resin) of 1.5 ml was pipetted into a 10 ml purification column. The resin was allowed to settle down completely by gravity (5-10 min). The supernatant was then aspirated gently. Followed by the addition of 6 ml sterile deionized water, the resin was resuspended by alternately inverting and gently tapping the column. The resin was again allowed to settle by gravity, and then the supernatant was aspirated gently. For purification under native conditions, 8 ml of native binding buffer was used. The resin was resuspended by alternately inverting and allowed to settle by gravity as described earlier, followed by aspiration of the supernatant gently.

Native purification of the soluble recombinant protein for activity determination

The supernatant of larger culture volume prepared as mentioned above was incubated with Probond resin for 2-4 hrs at 4^{o}C in the column. The protein was then packed into column and washed with 5 bed volumes of elution buffer and eluted as 0.5ml aliquots with a step gradient of imidazole (60mM, 120mM, and 200mM) in the same buffer (containing the Protease Inhibitor cocktail used as recommended by the manufacturer) and stored at –20^{o}C. The metal binding domain encoded by 6x-His-tag allows, purification of recombinant fusion protein by immobilized metal affinity chromatography (IMAC). Eluted fractions were checked by SDS-PAGE and Western blot as described above. To concentrate the eluate, dialysis was performed in a membrane (7cm) using 100,000 molecular weight cut-off, which could retain the protein of interest. 2 ml eluate fractions were collected and the pool fractions were taken in the semi permeable membrane with clampings on either end.

Dialysis was carried out against 500 ml of potassium phosphate buffer pH-7.8 for the first 4 hrs with moderate agitation at 4°C to remove the imidazole. Then fresh buffer was added and left overnight with a minimal agitation, the following day contents in the tubings were further concentrated in the presence of sucrose.

Estimation of protein and Heme assay

The protein content of crude lysate and eluate during purification process were estimated by Bichinchoninic acid method [25] following the manufacturer's protocol with BSA as the standard. The blue colour formation was based on reaction with proteins-amide group and copper sulphate solution, occurs after incubation at 37^{o}C for 30 min. The colour production was recorded spectrophotometrically at 562 nm. The absorbance of the final eluate was recorded at both 408nm for the heme protein (KatG) and 280 nm for the total protein, to determine the optical purity of the protein under study.

Activity gel assays

In order to qualitatively assess the catalytic capabilities of the enzymes, 10 ml of the induced culture was sonicated in the presence of ice-cold PBS and protease inhibitor and all the operations were carried out under cold condition. The sonicated lysates were centrifuged at 13,000 rpm and 4^{o}C for 30 min, and the supernatant were stored at -20^{o}C, required volume from the supernatant were subjected for native PAGE for activity analysis. Activity gel for catalase was performed using 7% native-PAGE in slab gels in Tris buffer system without SDS in the non-denaturing conditions. The samples were loaded along with the sample buffer in the ratio 4:1 without heating. The gel was run at 50 V at 4^{o}C to minimize heat generation. For analysis of catalase activity, following electrophoresis, the gels were soaked for 10 min in 30 mM H_2O_2 and staining solution consisting of 2% potassium ferricyanide and 2% ferric chloride [26].

Gels were stained for peroxidase activity using DAB as the electron donor species [27]. Gels were soaked in a solution containing 20 mg DAB in 30 ml of 50 mM potassium phosphate buffer pH 7.0 for 45 min, followed by a brief wash with water and then a second soak in the same buffer containing 20 mM H_2O_2. Peroxidase activity was visualized as brown bands on the gels. Colour development was usually complete within 30 min. Peroxidase-mediated oxidation of INH was visualized by soaking the gels in 200 ml of 50 mM potassium phosphate, pH 7.0, 274 mg INH, 50 mg NBT, and 60 ml of 30% H_2O_2. Colour development was usually complete between 30 min and 1 hr after which, gel was rinsed with distilled water and soaked in 7% acetic acid, 1% glycerol - overnight before mounting. Bands intensified slightly during incubation. Incubation of gels for 16 hrs in the staining solution from bands were much weaker than those seen using the complete stain of solutions [3].

Enzymatic activities

The activity of CP in the WT and mutant proteins were compared by measuring the decrease in H_2O_2 concentration at A_{240} for catalase. One unit of catalase is defined as the amount of enzyme that decomposes 1

µmol of H_2O_2 in 1 min at 37°C pH 7.0. Catalase activity as U/ml was determined from the slope of the plot representing O_2 evolution. Specific catalase activity was expressed as U/mg purified protein. The kinetic characterization using parameters such as K_m and *kcat* was determined with H_2O_2 at various concentrations *viz.*, 2-10 mM. The *Vi* was monitored on the basis of OD/min. V_{max} and K_m was found by MM graph and LB plot. **Peroxidase activity was determined using electron donors ABTS and INH Peroxidase activity was determined spectrophotometrically by ABTS method [28] with minor modifications. Assays were carried out in 1 ml final assay volumes containing 10 mM H_2O_2, 2.5 mM ABTS in 50 mM potassium phosphate pH 7.0. Aliquots of the appropriately diluted enzymes were added to initiate the reaction. Peroxidase activity was determined by measuring the oxidation rate of ABTS at A_{405} (ε_{405} = 36.8 mM^{-1} cm^{-1}). and kinetic parameters such as K_m and k_{cat} were determined as described above by including various concentrations of ABTS *viz.*, 0.5 mM, 1 mM, 1.5 mM, 2 mM and 2.5 mM. Peroxidase-mediated oxidation of INH was determined based on the methods developed by Shoeb *et al.*, for HRP [29]. Assays were carried out in 1 ml final volumes containing 9 mM INH; 0.2 mM NBT in 50 mM potassium phosphate pH 7.0 at RT. H_2O_2 was added to the reaction mixture finally. Aliquots of the appropriately diluted enzymes were then added to initiate the reaction. The activity was determined by measuring the reduction of NBT to formazan dye at A_{560} (ε_{560} = 15 mM^{-1} cm^{-1}). Kinetic characterization for the peroxidase activity with INH was also determined as described above with various concentrations of INH *viz.*, 1-9 mM.**

Statistical Analysis

The data obtained were from three independent experiments. Kinetic parameters *viz.*, k_{cat}, V_{max}, K_m values were calculated using MM and LB plots with the help of Graph Pad Prism software V-5.0.

RESULTS

To determine the functional differences between the WT and mutant KatG, WT *katG* gene with S315 from laboratory strain H37Rv and mutant *katG* gene containing the allele S315R from INH^r clinical isolate was cloned, overexpressed, purified and characterized, in this study. The amplified *katG* genes of 2.2 kb from strain H37Rv and mutant (Fig. 2A) were purified by Qiagen columns and cloned into the pBAD/Thio-TOPO cloning and expression vector, followed by the ligation reaction, they were successfully transformed in the competent host TOP10 cells using TSS buffer. The transformants were selected using standard methods of plating the cells on selection plates containing ampicillin and X-gal. Recombinant vectors were screened from the transformants by the isolation of plasmid DNA by alkai lysis method (Fig. 2B). Out of 22 colonies, which were screened two of them found to have the recombinant plasmid with insert

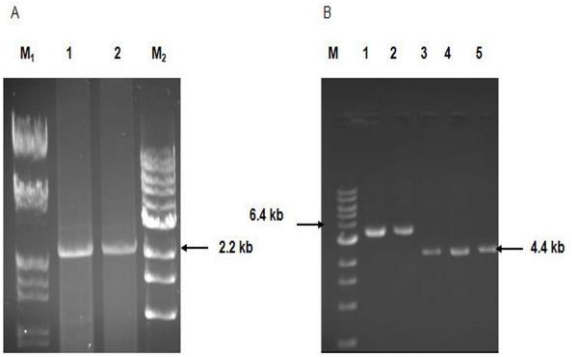

Fig. 2A: Amplification of *katG* gene Lane M : M_1- 23 kb ladder , M_2-1 kb DNA ladder, Lane 1 & 2 : Amplified products (WT and Mutant)

Fig. 2B: Isolation of recombinant plasmid DNA (pBAD-Thio TOPO) Lane M: 1 kb DNA ladder , Lane 1 and 2 : Recombinant plasmid DNA, Lane 3-5: Plasmid DNA without insert

determined on the basis of their size, the rest contain only the vector without insert. Of the two recombinant plasmids one showed the correct orientation of *katG* sequence downstream to the EK recognition site present in the vector which was determined by sequencing of recombinants in frame with the C-terminal peptide and also by restriction digestion by *NcoI*. After ensuring the presence of *katG* in forward direction, the specific transformant colony, which produced the recombinant plasmid, was propagated for further expression studies. Initially pilot expression studies like the time kinetics by the induction of L-arabinose were carried out for 1 to 5 hr. The protein profiles of un-induced and induced culture lysate with L-Arabinose (0.02%) were run in 10% SDS-PAGE and stained with CBB (Fig. 3A). Expression of the recombinant proteins was observed as early as 3 hrs, with maximal induction of protein when 0.2% L-arabinose was used. The size of the recombinant protein was found to be 96 kDa (13 + 80 + 3) 13 kDa for horseradish peroxide (HP) –thioredoxin fused with EK, 80 kDa for KatG and 3 kDa for the tag along with V5 epitope. The pBAD/Thio-TOPO system produces a recombinant protein with EK of 13 kDa as an N-terminal fusion partner of the cloned gene product (*katG*) in the center and a 6x-His-tag (3 kDa) as a C-terminal fusion partner. The purification of KatG proteins was carried from 500 ml of overnight grown culture containing 100 µg/ml ampicillin and 1 ml of 20% L-Arabinose. Recombinant proteins were obtained in the pellet fraction and purified proteins were eluted with 200 mM imidazole. The purity was confirmed both by SDS-PAGE (Fig. 3B) and Western blot. For the detection of KatG protein, the above SDS gel containing the protein profile was subjected to transfer on to nitrocellulose membrane to confirm the presence of the overexpressed protein.

ISSN: 0974-5343
IJMST (2011), 4(3):13-22

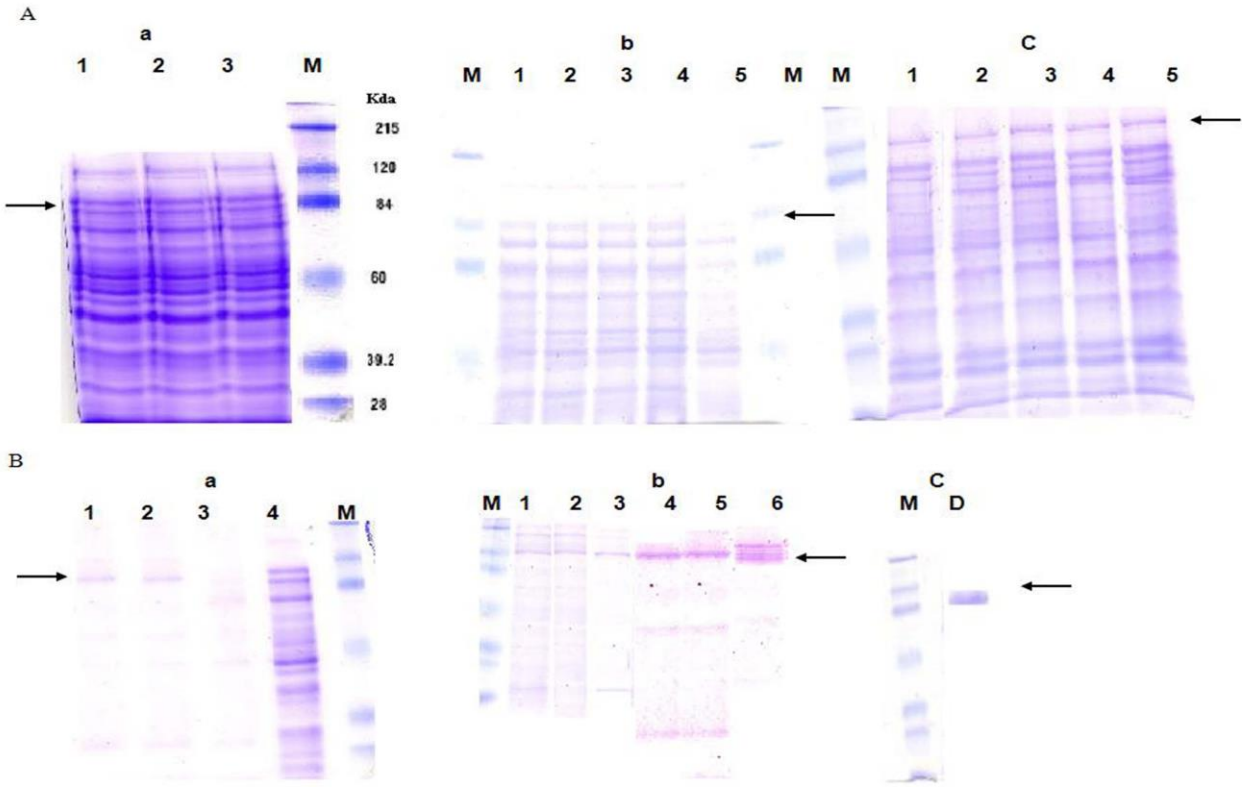

Fig. 3A: SDS-PAGE showing expression profiles of proteins (a) Lanes M: Broad range marker, 1-3: Protein profiles of un-induced culture Lysate (b) WT profile (c) Mutant profile. Lanes 1, 2, 3, 4, 5: 5hr, 4hr, 3hr, 2hr, 1hr induced culture.
Fig.3B: SDS-PAGE showing purified proteins (a) WT- Lanes 1-2: Eluates 3: Flow through 4: Crude lysate (b) Mutant -Lanes 1-2: Crude lysate 3: Flow through 4, 5, 6: Eluates (c) D: Dialysate Arrows indicate KatG protein of 97 kDa

The Western blotting (immunoblotting) was performed using anti-His-C-terminal-HRP antibody (Fig. 4A).

Protein concentration in mg/ml was determined in the crude lysate (4.5), flow through or the wash buffer (3.8), in eluate fractions-I (2.7), II (1.8), III (1.1) and dialysate (1.5) by bichinchoninic acid method. The optical purity ratio of the KatG proteins (Reinheitzahl or *Rz*, defined as A_{408}/A_{280}) was checked in the final eluant and was found to be 0.57 for the WT and 0.52 for the mutant.

DISCUSSION

The expression and purification of WT and mutant types of KatG in the present study was performed byrecombinant DNA technology pBAD/Thio-TOPO cloning and expression vector similar to a report [30]. To qualitatively visualize the function of KatG enzyme activity, gel assays were performed in this study. The catalase activity was evident by the production of white bands against the dark background in both the WT and mutant (Fig. 4B-I) as documented by Pym *et al*. (2002) [12], wherein white bands were seen in both the WT and mutants studied except for the mutant Thr275Pro. In a report, WT KatG showed the production of white bands [9]. In this study, the peroxidase activity produced brown bands with DAB in both the WT and mutant similar to studies [3, 11] for WT (Fig. 4B-II). Different activities (purple colour and colourless bands formation) were observed upon exposure to INH with NBT (Fig. 4B-III) in the WT and mutant which was supported by two of the reports [3, 10].

The quantitative analysis was carried out by spectrophotometric method. Catalase activity was assessed with H_2O_2 as substrate whereas peroxidase activity was dissected with substrates like ABTS, INH (Table 1). As KatG functions as a broad specificity peroxidase, oxidizing various electron donors, the oxidation rates of electron donors were measured using kinetic parameters. Kinetic characterization was implemented for the catalase activity with H_2O_2 whereas for peroxidase activity with ABTS and INH.

There was a sharp increase in the catalase activity curve for the initial 2 min followed by which the activity gradually decreases. The H_2O_2 concentration for the assay was standarized at 10 mM. The mutant showed less activity profile compared to WT. The activity of catalase in WT and mutant was estimated to be 115 and 79.2 U/mg respectively.

The peroxidase activity of the enzyme was tested with organic H donors, including INH, ABTS, utilizing

ISSN: 0974-5343
IJMST (2011), 4(3):13-22

H_2O_2 as oxidants for the peroxidatic reactions. Under the conditions of constant peroxide flux, consumption of O_2 in the reaction was increased corresponding to the rate of INH oxidation as evidenced by the reduction of NBT. Reduction of NBT to formazan dye by enzymatically oxidized radical species of INH was monitored spectrophotometrically. The activity monitored using ABTS showed an increase in the activity during the first 2 min, thereafter the activity was maintained in a decreased level. Moderate reduction was observed in the mutant compared to the WT KatG. Our results are analogous to those obtained

Table 1. Catalase and Peroxidase activities of WT and mutant

Enzyme	Specific activity (U/mg) ± SD	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1} s^{-1}$)
Catalase (H_2O_2)				
WT	115± 5.5	5641 ±2	7.6±0.2	7.4 ±0.03 x10^8
Mutant	79.2±19	5088 ±60	8.5 ±0.5	5.9 ± 0.05 x10^8
Peroxidase (ABTS)				
WT	0.15±0.025	6.7±0.3	0.93 ±0.2	7.2± 0.43 x10^8
Mutant	0.12±0.015	4.8 ±0.75	1.7± 0.2	2.8±0.3 x10^8
Peroxidase (INH)				
WT	0.19± 0.01	4±0.60	5.35 ±0.2	7.4± 0.26 x10^6
Mutant	0.13± 0.01	2.4±0.4	8.2 ±0.3	2.9 ± 0.1 x10^6

Specific activities were expressed as U/mg = micromoles/min^{-1}/ml^{-1} and were determined as the average of three experiments. k_{cat} is the enzyme turnover rate and is expressed on the basis of dimeric KatG native enzyme and mutant containing two active site

by [7], found decreased activity for the KatG mutants upon treatment with ABTS.

Catalase activities of KatG enzymes were determined at various concentrations of (2mM to 10mM) H_2O_2 (Fig. 5A). The MM graph and LB plot (Fig. 6A) were generated and values of K_m, k_{cat}, and k_{cat}/K_m are shown in Table-1. KatG from *M. tuberculosis* is a very efficient catalase with a K_m of 7.6 mM of H_2O_2 and a k_{cat} of 5461 x 10^6 s^{-1}. WT KatG displayed a k_{cat} K_m, and k_{cat}/K_m values relatively higher than the mutant. The KatG WT and the mutant catalase activity displayed a k_{cat}/K_m value of 7. 4 and 5.9 x 10^8 M^{-1} s^{-1} respectively. For the kinetic characterization of peroxidase activity with ABTS various concentrations of ABTS was chosen (Fig.5B and 6B). Compared to WT KatG, the peroxidase activity of ABTS in KatG mutant showed 29% lower k_{cat} value and 46% higher K_m value. The observed decreases in k_{cat}/K_m were primarily a consequence of higher K_m values.

Peroxidase activity varies as a function of INH concentration of 1mM to 9 mM and found to be optimum at 9 mM. The estimated K_m from the MM graph and LB plot (Fig. 5C and 6C) for WT was 5.35 mM with a k_{cat} of 4.0 s^{-1}. For the mutant under the same conditions, the K_m was 8.2 mM with a k_{cat} of 2.4

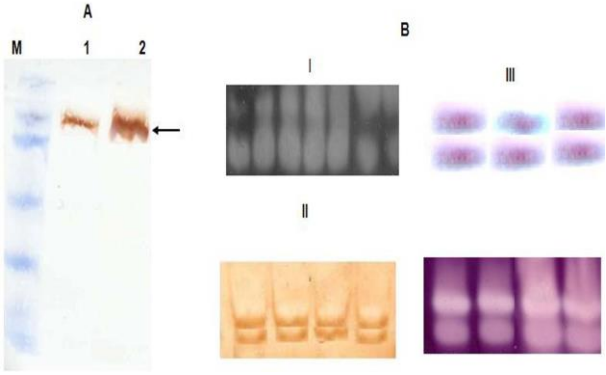

Fig. 4A: Western blot with Anti-His (C-term) antibody. Lane 1and 2: WT and mutant. Arrow indicate KatG protein of 97 kDa and M - Marker.
Fig. 4B: (I) WT and mutant displayed similar catalase activity [White bands] (II) WT and mutant displayed similar peroxidase activity [Brown bands] (III) WT displayed Peroxidase activity purple bands and mutant displayed colourless bands for upon treatment with INH.

s^{-1}. Thus WT has higher (40%) enzymatic affinity and catalytic turnover than the mutant. The mutant oxidizes INH at a slower rate whereas a recent report [9] has demonstrated that the mutant S315N completely lost their ability to convert INH into the InhA inhibitor.

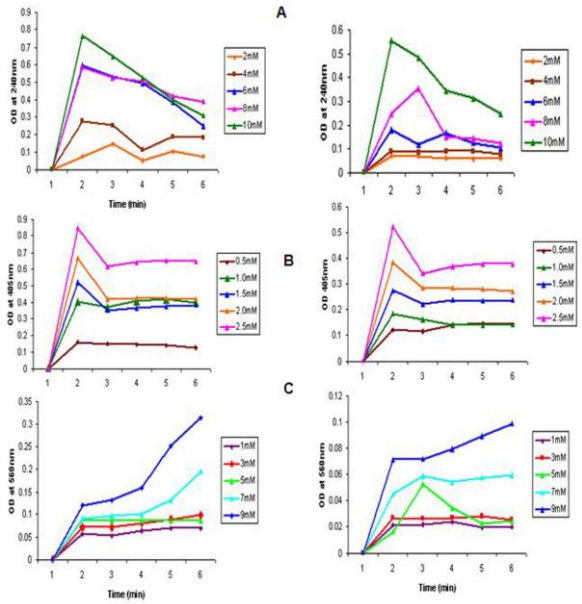

Fig.5: Effect of various concentrations of substrates for the determination of *Vi.* Catalase activity (A) H_2O_2, Peroxidase activity (B) ABTS (C) INH. WT: Left panel, Mutant: Right panel.

In summary, the catalase activity in the mutant was decreased by 32%. The activity of peroxides was decreased in the mutant by 20% for ABTS, 32% for INH, 88% compared to WT. The catalytic turnover (k_{cat}/K_m) in KatG mutant compared to WT KatG was decreased by 20% for catalase activity. The peroxidase activity showed 62% and 61% decrease with ABTS and INH respectively. Hence the kinetic data suggests that the mutant activity towards INH was reduced contributing to INH resistance.

ISSN: 0974-5343
IJMST (2011), 4(3):13-22

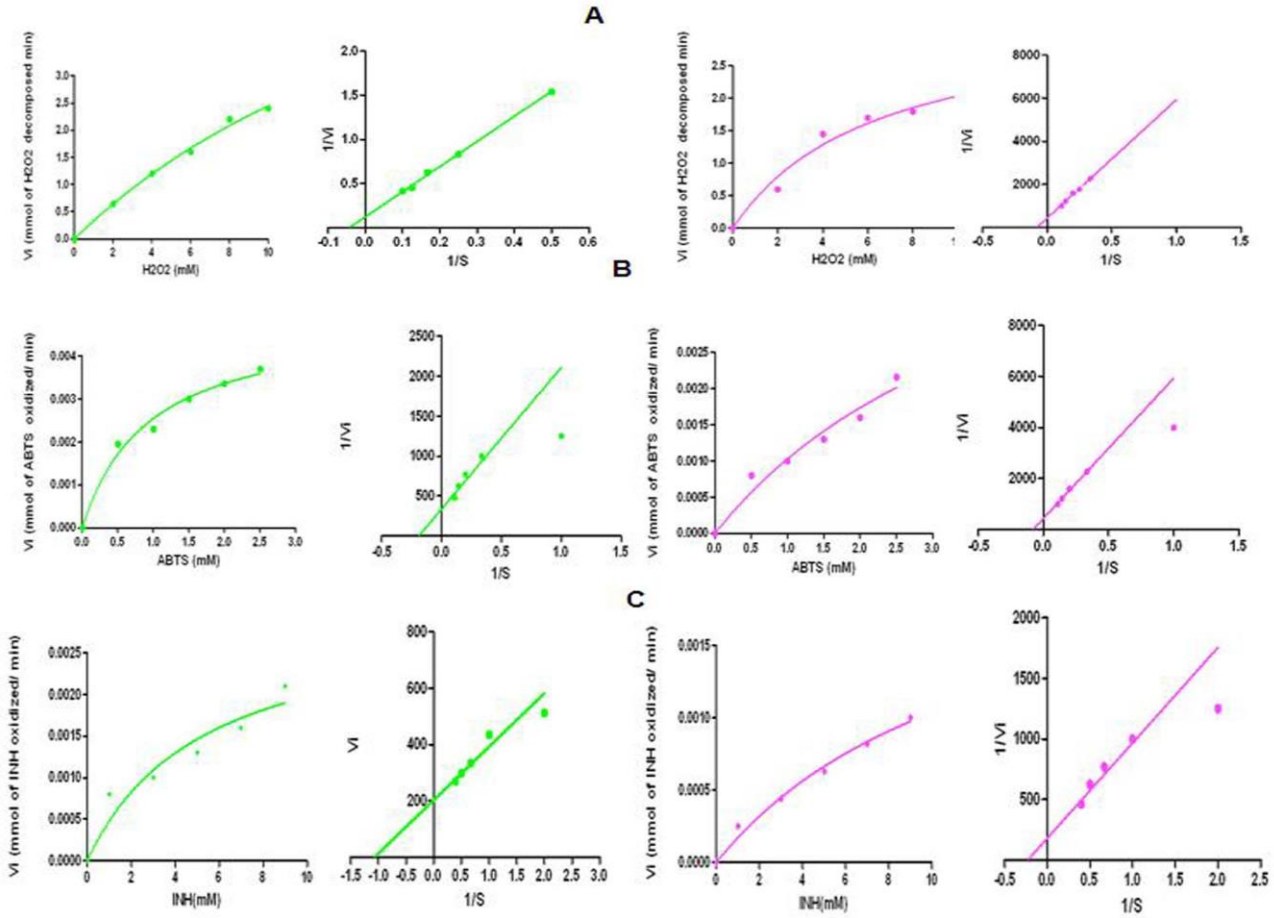

Fig. 6: MM graph (curve) and LB plot (linear) for the substrates (A) H_2O_2 (B) ABTS (C) INH. Left panel: WT, Right panel: Mutant

CONCLUSIONS

In this study, the functional basis of the WT and mutant was investigated which showed lower activity of the mutant compared to WT. The mutant's inability to function like WT with regard to oxidation of INH in addition to other substrates may be cause for resistance to INH. Overall suggesting that, in the mutant (S315R) the Ser to Arg conversion would have imposed some degree of steric impact on the structure of the mutant protein, rendering it inefficient to function like WT, perhaps due to the steric hindrance mediated by the bulkier guanido group present in the Arg residue instead of the simpler hydroxyl group in the Ser residue, which leads to altered binding of Heme subsequently, affects the binding of INH contributing to resistance.

ACKNOWLEDGEMENTS

Dr. A. Nusrath Unissa received financial support by Indian Council of Medical Research for Senior Research Fellowship.

CONFLICTS OF INTEREST

There is no conflict of interest among authors.

ABBREVIATIONS

ABTS	2,2- azinobis (3 ethylbenzothiazolinesulfonic acid)
BSA	Bovine serum albumin
CBB	Coomassie brilliant blue
CTAB	Cetyl triammonium bromide
DAB	Diaminobenzidine
EDTA	Ethylene diamine tetra acetic acid
H_2O_2	Hydrogen peroxide
HRP	Horse radish peroxidase
k_{cat}	Catalytic turnover
K_m	Michealis constant
MM	Michealis menten
NBT	Nitro blue tetrazolium salt
O_2	Oxygen
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
SDS	Sodium dodecyl sulphate
TAE	Tris acetate buffer
TSS	Transformation storage solution
Vi	Initial velocity
V_{max}	Maximum velocity
WT	Wild-type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyrinoside
ε	Extinction coefficient

REFERENCES

[1] Nachega JB, Chaisson RE (2003) Tuberculosis Drug Resistance: A Global Threat. Clin. Infect. Dis. [Suppl 1] 36 24-30.

[2] Middlebrook G. (1954) Isoniazid-resistance and catalase activity of tubercle bacilli; a preliminary report. Am Rev Tuberc. 69:471-472.

[3] Hillar A, Loewen PC (1995) Comparison of isoniazid oxidation catalyzed by bacterial catalase-peroxidases and horseradish peroxidase. Arch Biochem Biophys. 323,438-446.

[4] Zhang Y, Heym B, Allen B, Young D, Cole ST (1992) The catalase-peroxidase gene and Isoniazid Resistance of *Mycobacterium tuberculosis*. Nature 358, 591-593.

[5] Nagy JM, Cass AE, Brown KA (1997) Purification and characterization of recombinant catalase-peroxidase, which confers isoniazid sensitivity in *Mycobacterium tuberculosis*. J Biol Chem. 272,

[6] Johnsson K, Froland WA, Schultz PG (1997) Over expression, purification, characterization of the catalase-peroxidase *katG* from *Mycobacterium tuberculosis*.J Biol Chem. 272:2834-2840.

[7] Rouse DA, DeVito JA, Li Z, Byer H, Morris SL (1996) Site-directed mutagenesis of the *katG* gene of *Mycobacterium tuberculosis*: effects on catalase-Peroxidase activities and isoniazid resistance Mol Microbiol. 22,583-592

[8] Ramaswamy S, Musser JM. (1998) Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. Tuber Lung Dis. 79, 3-29.

[9] Wei CJ, Lei B, Musser JM, Tu SC (2003) Isoniazid activation defects in recombinant *Mycobacterium tuberculosis* catalase-peroxidase (KatG) mutants evident in InhA inhibitor production Antimicrob Agents Chemother. 47,670- 675

[10] Saint-Joanis B, Souchon H, Wilming M, Johnsson K, Alzari PM, Cole ST (1999) Use of site-directed mutagenesis to probe the structure, function and isoniazid activation of the catalase/peroxidase, KatG, from *Mycobacterium tuberculosis* Biochem J. 338, 753-760

[11] Joseph DeVito A, Sheldon Morris (2003) Exploring the Structure and Function of the Mycobacterial KatG Protein Using trans-Dominant Mutants. Antimicrob Agents Chemother. 47:188-195.

[12] Pym AS, Saint-Joanis B, Cole ST (2002) Effect of *katG* mutations on the virulence of *Mycobacterium tuberculosis* and the implication for transmission in humans. Infect Immun Infect Immun. 70, 4955-4960

[13] Yu S, Chouchane S, Magliozzo RS (2002) Characterization of the W321F mutant of *Mycobacterium tuberculosis* catalase-peroxidase KatG Protein Sci. 11, 58-64

[14] Saint-Joanis B, Souchon H, Wilming M, Johnsson K, Alzari PM, Cole ST (1999) Use of site-directed mutagenesis to probe the structure, function and isoniazid activation of the catalase/peroxidase, KatG, from *Mycobacterium tuberculosis* Biochem J. 338,753-760

[15] Wengenack NL, Todorovic S, Yu L, Rusnak F (1998) Evidence for differential binding of isoniazid by *Mycobacterium tuberculosis* KatG and the isoniazid-resistant mutant KatG(S315T) Biochemistry 37,15825-15834

[16] Wengenack NL, Uhl JR, Stamand AL, Tomlinson AJ, Benson LM, Naylor S, et al. (1997) Recombinant *Mycobacterium tuberculosis* KatG (S315T) is a competent catalase-peroxidase with reduced activity toward isoniazid J Infect Dis.176:722-727

[17] Wengenack NL, Rusnak F (2001) Evidence for isoniazid-dependent free radical generation catalyzed by *Mycobacterium tuberculosis* KatG and the isoniazid-resistant mutant KatG(S315T) Biochemistry 40:8990-8996

[18] Wengenack NL, Lopes H, Kennedy MJ, Tavares P, Pereira AS, Moura I, et al. (2000) Redox potential measurements of the *Mycobacterium tuberculosis* heme protein KatG and the isoniazid-resistant enzyme KatG (S315T): insights into isoniazid activation. Biochemistry 39, 11508-11513

[19] Marttila HJ, Soini H, Eerola E, Vyshnevskaya E, Vyshnevskiy BI, Otten TF, et al. (1998) A Ser315Thr substitution in KatG is predominant in genetically heterogeneous multidrug-resistant *Mycobacterium tuberculosis* isolates originating from the St. Petersburg area in Russia Antimicrob Agents Chemother.42, 2443-2445

[20] van Soolingen D, de Haas PE, van Doorn HR, Kuijper E, Rinder H, Borgdorff MW (2000) Mutations at amino acid position 315 of the *katG* gene are associated with high-level resistance to isoniazid, other drug resistance, and successful transmission of *Mycobacterium tuberculosis* in the Netherlands J Infect Dis.182,1788-1790

[21] Suarez J, Ranguelova K, Schelvis JP, Magliozzo RS (2009) Antibiotic resistance in *M. tuberculosis*: Peroxidase intermediate bypass causes poor isoniazid activation by the Ser315Gly mutant of *Mycobacterium tuberculosis* catalase-peroxidase (KatG). J Biol Chem. 284,16146-16155

[22] Siddiqi N, Shamim M, Hussain S, Choudhary RK, Ahmed N, Prachee, et al. (2002) Molecular characterization of multidrug-resistant isolates of *Mycobacterium tuberculosis* from patients in North India. Antimicrob Agents Chemother. 46,443-450

[23] Birnboim HC, Doly JA (1979) Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA. Nucleic Acids Research. 7:1513-1523.

[24] Sambrook J, Fritsch E, Maniatis T. Molecular Cloning: A Laboratory Manual. In: Harbor CS, eds. 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989.

[25] Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, et al. (1985) Measurement of protein using bicinchoninic acid. Anal Biochem. 150, 76-85

[26] Woodbury W, Spencer AK, Stahman MA (1971) An improved procedure using ferricyanide for detecting catalase isozymes. Anal Biochem. 44,301-305

[27] Gregory EM, Fridovich I (1974) Visualization of catalase on acrylamide gels. Anal Biochem. 58:57-62.

[28] Smith AT, Santama N, Dacey S, Edwards M, Bray RC, Thorneley RN (1990) Expression of a synthetic gene for horseradish peroxidase C in Escherichia coli and folding and activation of the recombinant enzyme with Ca+ and heme. J Biol Chem. 265:13335-13343

[29] Shoeb HA, Bowman BU Jr, Ottolenghi AC, Merola AJ. (1985) Peroxidase-mediated oxidation of isoniazid. Antimicrob Agents Chemother.27,399-403

[30] Wang RF, Wennerstrom D, Cao WW, Khan AA, Cerniglia CE (2000) Cloning, expression, and characterization of the katG gene, encoding catalase-peroxidase from the polycyclic aromatic hydrocarbon-degrading bacterium Mycobacterium sp. Strain PYR-1. Appl Environ Microbiol. 66, 4300-4304